Original Article

Development of NT3 Genetically Engineered cells using human Lymphocytes.

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Abstract Objective To establish a lymphocyte line capable of long survival and expressing human NT-3 to lay a foundation for future animal and human cochlear gene transfection research. **Methods** We collected lymphocytes from normal human blood via Ficoll fluid and added IL-2 into the serum culture medium to promote lymphocyte growth. The NT3 cDNA was obtained by RT-PCR and ligated with the eukaryon vector which is pIRES-DsRed2 using T4DNA enzyme. The NT3 cDNA gene was transfected into the lymphocyte line using cationic liposome (LP2000). The lymphocytes transfected with NT3-cDNA were examined by RT-PCR and Western-blot methods. **Results** We established a new method to extend in vitro lymphocytes survival time and to transfect NT3 into lymphocytes. The genetically engineered lymphocytes were capable of surviving over relatively long time. Positive protein signals were obtained by Western blot. **Conclusions** Using lymphocytes as the intermediary, recombined plasmid pIRES-DsRed2- NT3 is used to establish a lymphocyte line that expresses and secretes NT3. This cell line can be used in future animal gene cochlear transfection research and may help find an intermediary cell line for gene therapy for human deafness.

Key words Neurotrophin-3, Gene clone, Lymphocyte, Transfection

Neurotrophic factor is very important for development and functional maintenance of the nervous system. It is closely involved in neural injury and disease. It not only plays an important role in the maintenance of normal formation and regulation of physiological and biochemical functions of the inner ear auditory epithelium and neurons during embryonic development and after birth, but also participates in protecting and restoring functions in a number of acquired disease conditions of the auditory neuron.

NT3 is a protein family composed by 774 amino acid residues which are related closely with neurological structure and function. It has been shown that NT3 has a significant protective effect for the cochlea, but it has a short half-life. The specific anatomy of the cochlea has limited the application of NT3 in the cochlea. It would be necessary to repeatedly perfuse the cochlea in order to maintain an effective concentration inside the cochlea, which would inevitably cause damage to the cochlea. Transfection of a target gene into the cochlea therefore represents the only pathway which may provide a long-term supply of the protein factor and maintain a long-term efficacy of treatment [1-6].

There is no specific treatment for sensorineural hearing loss at present. Recent research has started to focus on gene therapy and stem cell transplantation. In both clinical and experimental research, gene transfer is an exciting new instrument, which is gaining increasing application in research on preventing and restoring sensorineural hearing loss in recent years. Transgenic expression is helpful in detecting genes playing a special role in cochlear cell biology. Such research not only increases understanding of the pathophysiology of deafness, but may also provide gene therapy for inner ear disease [7,8].

With the progress of inner ear gene therapy study, it

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has become possible to transfect a target gene into the cochlea through a gene vector, although the foreign carrier may potentially harm the host. We chose human lymphocytes as a carrier of the intermediate host because they are the major cellular components of the endo-and perilymphatic and cerebrospinal fluid. In case genetically engineered cells die in the host body, they will be removed by the immune cells, so there will be no unknown hazards by the lymphocytes.

The purpose and the significance of this study is that a stable genetically engineered cells in the form of the normal lymphocyte that can express and secret NT3 will be useful in finding an effective way of transferring genes into the cochlea, which is the foundation for future clinical application of gene transfer therapy.

Materials and methods

1.1 *Materials* Human fetal liver tissues were collected from fresh liver specimen in aborted fetus. The eukaryon vector was purchased from Invitrogen Corporation, USA. Restriction enzyme EcoRI and BamHI as well as T4DNA ligase enzyme were purchased from Takara Corporation (Japan). Using the human NT3 sequences in the genebank (log No, 002527), the 5, end with a EcoRI and BamHI restriction sites of PCR primers wsa designed, and the primers were synthetized by the Bioasia Corporation, Shanghai. Recombined eukaryon expression vector pIRES-DsRed2-NT3 was cloned and constituted in our laboratory. Human lymphocytes were obtained from the Nanjing Blood Center. Recombined Human IL-2 was purchased from R&D Systems, USA. Lipofectamine 2000 trasfection kit, fetal calf serum and RMPI1640 powder were purchased from the Gibco Corporation. NT3 antibody was purchased from the Chemicon Corporation. The ECL LED system was purchased from the Pierce Corporation.

1.2 *Methods*

1.2.1 Clone of human NT3 gene The total RNA was isolated from fresh fetal liver by the Trizol method and reverse transcribed into cDNA (with random hexmer primers). The NT3 gene was amplified by PCR with a primer at 55℃ over 30 cycles. A fragment of about 774 bp from the PCR products were isolated by electrophoresis with 1% Agarose gel and purified by gel extraction kit. Both the purified NT3 PCR production and eukaryotic expression vector pIRES-DsRed2 were digested with EcoRI and BamHI. Then T4DNA ligase was applied to the digestion mixture for 4 hours and the success of ligation was verified by gel electrophoresis and enzyme digestion.

1.2.2 cultivation of human lymphocyte

A Cell extraction Equal volume of concentrated white blood cell and saline was mixed and the mixture was applied to Ficoll. After centrifuge for 25 min by 2500 rpm, the mononuclear cell layer (white membrane layer) was transferred by moving into a new tube.

B Cell washing The cells were washed by saline and then centrifuged for 5 min at 1500 rpm. After removing the supernatant, the cells were suspended with RMPI1640.

C Cell culture The isolated cells were cultured with 10%FCS RMPI1640 at the cell density of 2×10^6 cells / ml, with Il-2 at 37 oC, 5% CO_2.

1.2.3 Gene transfection

Lymphocytes were transfected when cell density reached 80% . DNA-Lipofectamine 2000 complexes were prepared as following: 16 ug pIRES-DsRed2 plasmid DNA was applied to 800 ul of growth medium without serum. 48 μl of Lipofectamine 2000 was diluted with 800 μl growth medium without serum and incubated for 5 minutes at room temperature. Then the diluted Lipofectamine was applied to plasmid DNA. After incubation for 20 minutes at room temperature, the DNA-Lipofectamine 2000 complexes were applied to cultured Lymphocytes and incubated at 37℃ in a CO_2 incubator for 48 hours. Transfection was verified by examining green fluorescence under a fluorescence microscope.

1.2.4 Transfection confirmation The transfection in human lymphocytes was verified by RT-PCR.

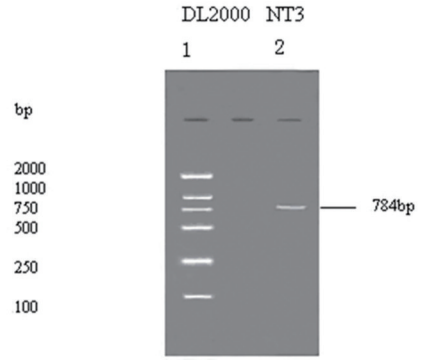
1.2.5 Western Blot analysis Total protein from untransfected human lymphocytes, transfected human lymphocytes with NT3 or vector were isolated. 30 ug of protein from each sample was separated by SDS -polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with skimmed milk, anti-NT3 antibodies was added to the membrane and incubated. Then horseradish peroxidase (HRP) labeld second antibody and ECL were applied to the membrane

for X-ray film exposure.

Results

2. 1　Figure 1 shows the NT3-cDNA production abstracted from human fresh fetal liver tissue.

2.2　*Sequencing* the 774bp fragment coding region numbered "2" and "3" in the NT3-cDNA coincided with



1. Standard MMOL of nucleic acid; 2. RT-PCR product from human liver (784bp)

Figure 1 RT-PCR product from human liver

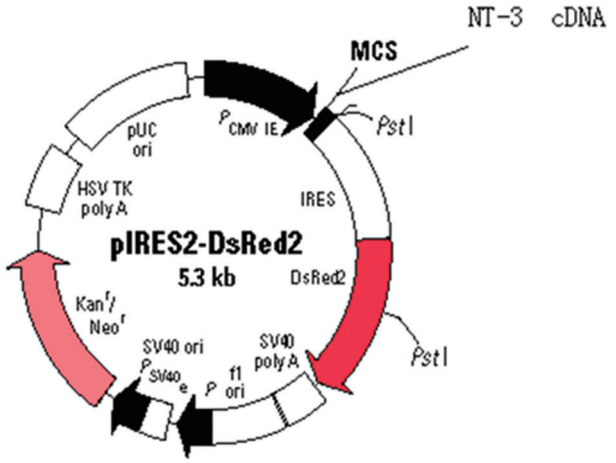


Figure 2 Construction of the pIRES-DsRed2-NT3 vector

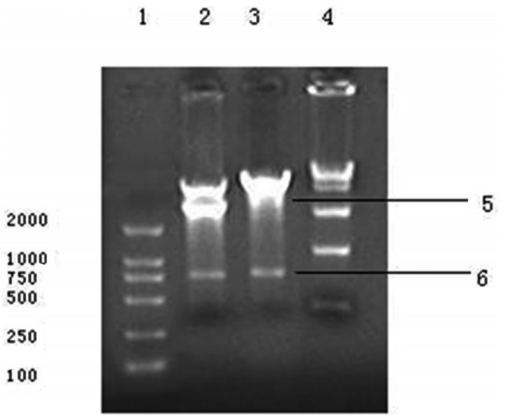
the published sequence number "002" and "527".

2.3　Figure 3 shows electrophoresis results of constitution of recombined plasmid pIRES-DsRed2-NT3.

2.4　Human lymphocytes grew well and survived at last 3 weeks on on medium containing fetal calf serum and interleukin -2 (IL-2) (Figure. 4). Figure 5 shows lymphocytes transfected with NT-3 under a fluorescence microscope.

2.5　*RT-PCR production*

Following lymphocyte transfection with NT3 and PCR reaction, electrophoresis showed a 774 bp fragment, but



1 Standard MMOL of nucleic acid; **2** RT-PCR product from human liver (784bp)

Figure 3　Analysis of pIRES-DsRed2-NT3 by enzyme digestion

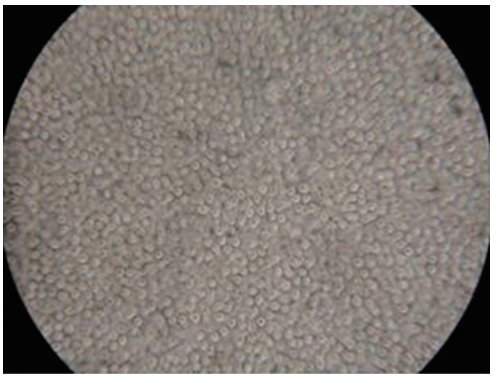


Figure 4　Culture of human Lymphocytes

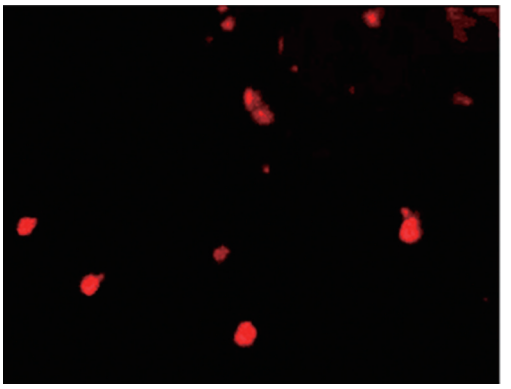
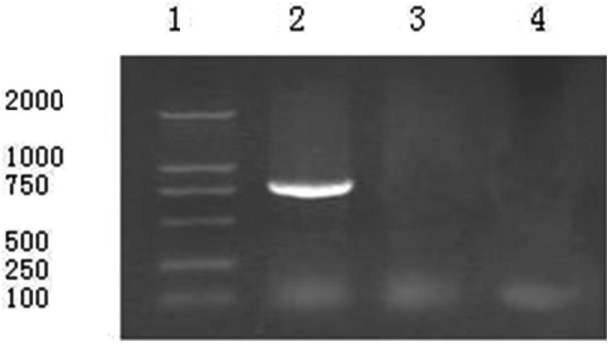


Figure 5　Detection of Lymphocytes transfected with pIRES-DsRed2-NT3

not in cells transfected with empty vectors or in non-transfected human lymphocytes (Figure. 6).

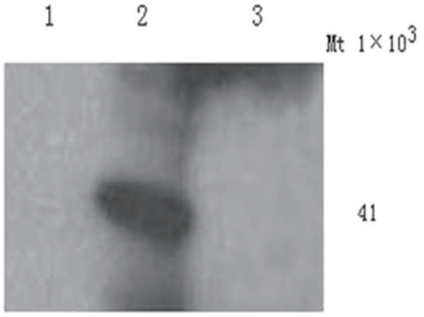
2.6　*Western Blot analysis*

There was a clear protein band in the 2nd lane (human lymphocytes transfected with NT3 gene) on the



1. Marker; **2.** RT-PCR product from Lymphocytes transfected with NT3-cDNA (784bp); **3.** Lymphocytes transfected with pIRES-DsRed2-NT3; **4.** untransfected lymphocytes.

Figure 6 RT-PCR product from three types of Lymphocytes



1. from cells transfected with pIRES-DsRed2-NT3;
2. from cells transfected with pIRES-DsRed2-NT3; 3:from untransfected cells.

Figure 7 Western blot assay for expression of NT3 protein

X-ray film, with a relative molecular mass of 41×10^3, which was consistent with NT3 protein molecular mass. In contrast, no positive protein band was seen in lanes corresponding to cells transfected with empty vector group and non-transfected human lymphocytes. The results indicate that human lymphocytes transfected with NT3 have the ability to secret active protein.

Discussion

Neurotrophin-3 is a group of family protein closely related to neural structure and function, regulating the development, growth, differentiation and survival of the neuron. It is not only important for maintaining the survival of auditory epithelial cells and auditory neurons, but also the most basic nutritional factors for the development of inner ear [9].

Auditory signals are formed and transmitted from hair cells in the auditory receptor organ of Corti to the brain via the spiral ganglion. Damage or loss of hair cells or spiral ganglion neurons can cause hearing damage, which is usually permanent.

NT3 has been confirmed to be an important material for maintenance of survival of auditory epithelial and auditory neurons by in vivo and vitro studies. it has been shown that giving NT3 protein factor through microinjection or micro-osmotic pump can provide protective and therapeutic effects for sensorineural hearing loss [2,10-12].

However, the current administration routes are not suitable for long-term application, as maintaining a high level of expression of neurotrophic factors in the cochlea is critical for treatment success. Cochlea is a special sensory organ. Derby and Stover injected viral vectors carrying a reporter gene into the guinea pig cochlea and found no change in hearing function and cochlear morphology. Gene transfer into the cochlea is considered the only way to provide long-term treatments [4, 13-14].

The vector systems for gene transfection include viral and non-viral vector system. While the former carries relatively high transfection efficiency, its target genes introduce and integrate into the host DNA molecule directly. Therefore, its safety remains to be determined. The advantages of non-viral vector is that it is safe and easy to conduct, although its biggest problem is the low efficiency of transfection.

In this study, we selected liposome LP2000 as the gene vector, which has low toxicity and high transfection efficiency and expression levels in various cells. It demonstrates relative high transfection efficiency under both serum and non-serum conditions [4].

At the same time we choose lymphocytes as the host cell for target genes to express and secrete transfected genes. Such studies usually involve in vitro experiments because human lymphocytes can be cultured in less than 1 week. We have established experimental conditions that allow us to maintain lymphocytes for 3 weeks while retaining biological activity. And we continue to search for favorable experimental conditions to increase the life cycle of lymphocytes. The current study shows that human lymphocytes transfected with pIRES-DsRed2-NT3 could survive under experimental

conditions. This represents a useful tool for future gene transfection studies.

The most important feature and innovation of the current study is to use the lymphocyte as the intermediate host of target gene. Transfer via foreign carriers, especially the viral vector, into functional cells directly may cause potential harm[15]. Direct viral vectors transfer into the body may activate the body´s immune system quickly to remove these viral vectors, particularly involving the complement system [16].

Lymphocytes are the main cellular components in the cerebrospinal fluid and endo- and perilymphatic fluids. They are the ideal cells for transplantation of genetically engineered cells in the cochlea. Transplantation of genetically engineered lymphocytes into the target organs in vivo avoids integration of foreign carriers integrate into functional cells. If genetically engineered lymphocytes die, they can be removed by the body´s immune system without the possible harm foreign carriers, especially viral vectors, may produce.

In earlier studies, Gao Xia etc. have reported successfully recombining and transfecting NT3 gene and pCD vector into Jurket lymphoma cells, which can easily survive in vitro experiments, to produce genetically engineered Jurket lymphoma cells with biological activity. Studies indicate that intrathecal injection of NT3 delays aging of cochlear function in inbred mouse [17, 18]. Establishing a genetically engineered lymphocyte line may be a safe and effective approach to gene therapy for sensorineural hearing loss.

In this study, our goal is to produce biologically active NT3-engineered cells using primary cultured human lymphocytes as the transfection target. Our results show that this is feasible and the resultant cells are capable of expressing NT3 and biologically active. This work has laid the foundation for future animal studies.

For future research, we plan to build genetically engineered lymphocytes in guinea pigs, transplant the cells into the cochlea, and study effects of transplanted cells on hearing improvement and corresponding morphological changes in guinea pigs with hearing loss.as well as on cochlear homeostasis. We can assume boldly that it is not impossible to transplante human autologous genetically engineered lymphocytes to treat hearing impairment in the future.

Acknowledgments

We thank Wandong She, Yanhong Dai and Feng Chen for technical help. This work was supported by National Natural Science Foundation of China(30973302), Medical Important People Project of Jiangsu Province (RC2007010) and Medical Important Developing Project of Nanjing (ZKX06019).

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(Received May 4, 2010)